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Award Number: W81XWH-06-1-0532

TITLE: The Impact of the 6:3 Polyunsaturated Fatty Acid Ratio on Intermediate Markers of Breast Cancer

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REPORT DATE: May 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
04-05-2008	Annual summary report	*20-Apr-2007 to 19-Apr-2008*

4. TITLE AND SUBTITLE
The impact of the 6:3 polyunsaturated fatty acid ratio on intermediate markers of breast cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0532

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alana Hudson

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Evidence suggests omega-6 (n-6) polyunsaturated fatty acids (PUFAs) promote breast cancer whereas omega-3 (n-3) PUFAs inhibit breast cancer growth. These fatty acids may influence breast cancer development by impacting prostaglandin E2 (PGE2) formation and consequently estradiol synthesis. We sought to establish the relationship between erythrocyte n-6 and n-3 PUFAs with serum estradiol and breast density, two hormonally-related breast cancer risk factors. We hypothesized that n-6 PUFAs and the 6:3 PUFA ratio are positively related and n-3 PUFAs negatively related to both risk factors. Nonsteroidal anti-inflammatory drugs (NSAIDs) also inhibit PGE2 formation, therefore we further hypothesized that estradiol levels would be lower among NSAID users. Participants (n=260) were eligible for these analyses if they were cancer-free, postmenopausal and not taking exogenous hormones. Estradiol levels were significantly lower among current users of NSAIDs as compared to non-users of NSAIDs. Further, estradiol concentration decreased with increasing total n-3 PUFAs and rose with increasing total n-6 PUFAs and the 6:3 PUFA ratio; however, this was noted only among non-users of NSAIDs. No relationship was observed between any fatty acid measure and breast density. In summary, lowering n-6 intake, increasing n-3 intake, or taking a NSAID may result in reduced estradiol synthesis and potentially breast cancer risk.

15. SUBJECT TERMS
omega-3 fatty acids, omega-6 fatty acids, estradiol, breast density, nonsteroidal anti-inflammatory drugs

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	45	19b. TELEPHONE NUMBER *(include area code)*
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1 Introduction

Experimental evidence suggests that omega-6 (n-6) polyunsaturated fatty acid (PUFA) intake promotes breast cancer growth (1), whereas consumption of omega-3 (n-3) PUFAs inhibits the development of this disease (2). Furthermore, it appears that the cancer promoting activity of the n-6 fatty acids is abrogated by the competitive inhibition of n-3 fatty acids (3, 4). The mechanism by which these fatty acids impact breast cancer development is unknown; however, experimental evidence indicates that these two families of fatty acids may influence risk by impacting eicosanoid synthesis. Specifically, when n-3 PUFAs displace n-6 PUFAs, prostaglandin E2 production (PGE2) is reduced, resulting in decreased aromatase activity and ultimately suppression of estrogen synthesis (**see Figure 1**). Whether the increase in PGE2 can cause substantial affects on circulating estradiol levels or localized estradiol levels in breast tissue remains undetermined. Therefore, utilizing fatty acids in erythrocytes as a biomarker of recent dietary intake, we sought to determine whether erythrocyte n-6 and n-3 fatty acids were associated with two postmenopausal breast cancer risk factors, circulating estradiol levels and percent mammographic breast density. We hypothesized that n-6 fatty acids and the 6:3 PUFA ratio are positively related and n-3 fatty acids negatively related to both risk factors. Moreover, because nonsteroidal anti-inflammatory drugs (NSAIDs) also inhibit PGE2 formation (**see Figure 1**), we further hypothesized that estradiol levels would be lower among NSAID users. NSAID data were not available at the time of mammogram; hence the relationship between NSAID use and mammographic density could not accurately be assessed. The study objectives were assessed by undergoing an ancillary study within the Mammograms and Masses Study (MAMS), a case-control study of estrogen metabolites, mammographic density and breast cancer risk. To be eligible for the ancillary study, participants were required to be breast cancer-free, postmenopausal, and not using hormone therapy, corticosteroids, or selective estrogen receptor modulators (SERMs) at study enrollment. 260 women were found eligible for these ancillary studies.

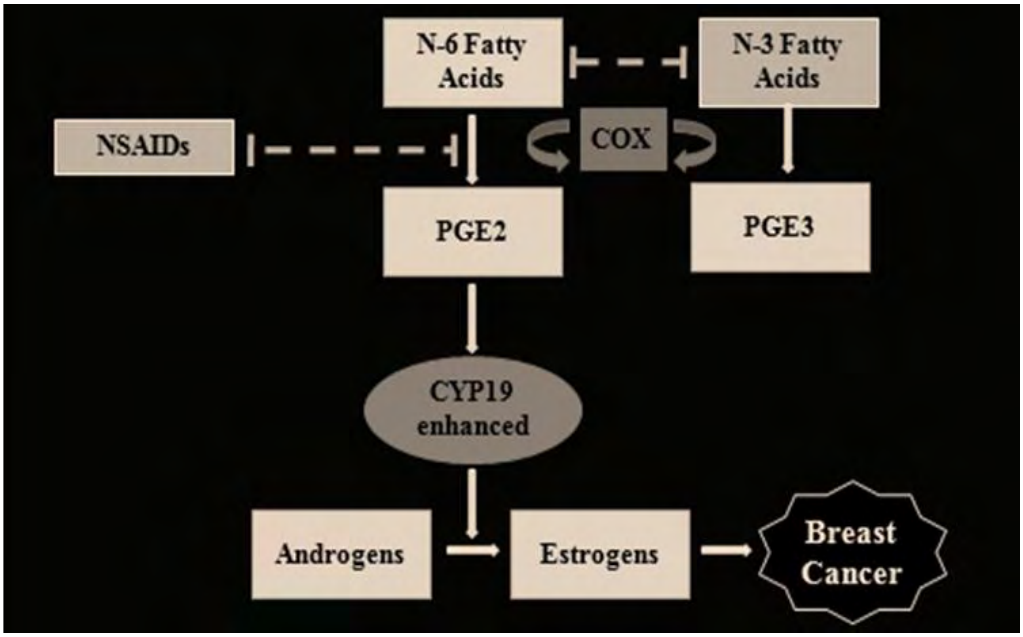

Figure 1. Biological model

2 Body

2.1 Research Activities

Review of year 1:

Key research accomplishments of the first year of work (months 1-12) included identifying the study population, obtaining biological specimens and mammographic films on study participants, and shipping biological specimens and mammograms for analysis. Additionally, ahead of schedule, we received laboratory data and density measurements, and reported on preliminary statistical analyses that assessed the relationship between fatty acids and circulating estradiol levels.

We are happy to report that we have made significant progress, during the last 12 months (months 13-24), in this ongoing grant. During this phase of funding the primary goals, which were not accomplished ahead of schedule in year 1, were to create a master data file and to perform final statistical analyses. The progress for year 2 is described as follows:

Creating a master data file:

A master dataset used for all statistical analyses was created by merging laboratory (estradiol and fatty acids) and density files with a study population demographics data file.

Final statistical analyses:

Omega-6 and omega-3 polyunsaturated fatty acids and estradiol analysis:

We have extended our preliminary analysis on which we reported in our first annual summary report. The finalized analyses resulted in a paper, which is currently under co-author review and will be submitted to a peer reviewed journal sometime in the next 1-4 months. A brief account of this analysis is given below and the most current version of the paper is included in the **Appendix**.

The goal of this analysis was to determine if a relationship existed between erythrocyte n-6 and n-3 fatty acid measures and postmenopausal serum estradiol levels. Participants in this cross-sectional analysis included 260 cancer-free postmenopausal controls enrolled in the MAMS. Only participants not reporting current use of hormone therapy, antiestrogens or corticosteroids at blood draw were included in the present analysis. Erythrocyte fatty acids were measured by gas chromatography at the University of Pittsburgh's Heinz Laboratory. Estradiol was measured in serum using an indirect radioimmunoassay, and values were logarithmically transformed to obtain normal frequency distributions. Multivariate associations for serum total estradiol according to tertile of fatty acid were assessed by analysis of covariance (ANCOVA). Adjusted geometric mean estradiol and 95% confidence intervals (CI) were calculated using least squares means, controlling for the effects of age (continuous), BMI (continuous), years menopausal (continuous), regular alcohol consumption in the past year (0g/day, <12g/day, ≥12g/day, entered as a dummy variable), and current smoking status (nonsmoker vs. smoker). The comparisons were adjusted for these variables as they were strongly related to the majority of fatty acid measures and serum estradiol levels. The geometric mean concentrations of estradiol were calculated by taking the anti-log of the least squares means after adjustment. NSAIDs reduce PGE2 synthesis and hence aromatase activation, thus, intake of the n-6 and n-3 PUFAs may have less of an effect on estradiol levels among NSAID users. Therefore, we

performed analyses stratified by NSAID use to assess possible effect modification by this variable.

Estradiol and fatty acids among NSAID non-users: Among non-users of NSAIDs, multivariate adjusted analyses revealed significant and borderline significant associations between fatty acid measures and estradiol. Total n-6 fatty acids was positively and significantly related to serum estradiol (lowest tertile=16.0pmol/L [95%CI: 13.4, 18.9] upper tertile=21.8pmol/L [95%CI: 18.3, 26.1]; p trend=0.02). On the contrary, geometric mean serum estradiol levels decreased with increasing tertile of total n-3 fatty acids (lowest tertile=24.3pmol/L [95%CI: 20.1, 29.3] upper tertile=18.4pmol/L [95%CI: 15.2, 22.3]; p=0.05). The total 6:3 PUFA ratio was positively related to estradiol and the finding approached statistical significance (lowest tertile=17.6pmol/L [95%CI: 14.6, 21.2] upper tertile=22.9pmol/L [95%CI: 19.0, 27.8]; p trend=0.06).

Estradiol and fatty acids among NSAID users: Among women reporting current NSAID use, no association was found between total n-6 fatty acids, total n-3 fatty acids, or the total 6:3 PUFA ratio and estradiol levels.

In summary, among women not reporting current NSAID use, we found a positive association between total n-6 fatty acids and estradiol and an inverse association between total n-3 fatty acids and estradiol. We further observed a positive relationship between the total 6:3 PUFA ratio with serum estradiol. Possibly through the reduced action of PGE2 on aromatase, low n-6 and high n-3 intake may reduce serum estradiol concentrations. As high postmenopausal circulating estradiol concentrations are related to increased postmenopausal breast cancer risk, these findings are consistent with the hypothesis that n-6 fatty acids increase the risk of breast cancer and n-3 fatty acids protect against this disease. However, similar associations were not noted among women who reported current NSAID use. A potential explanation for the null finding between total n-6 fatty acids and estradiol among NSAID users is that since NSAIDs inhibit PGE2 formation, limiting the amount of substrate available (n-6 fatty acid arachidonic acid) for PGE2 synthesis is not of biological importance. In addition, a relationship between total n-3 fatty acids and estradiol might not have been observed among NSAID users, because both n-3 consumption and NSAID use reduce PGE2 production, and therefore exposure to both anti-inflammatory agents might not offer additional benefit.

In conclusion, this study provides modest evidence supporting a positive association between n-6 PUFAs and the 6:3 PUFA ratio and a negative association between n-3 PUFAs and serum estradiol levels among women not using NSAIDs. Because circulating postmenopausal estradiol concentrations are causally related to breast carcinogenesis, these findings provide a mechanism through which the n-6 and n-3 PUFAs may alter breast cancer risk.

NSAIDs and estradiol analysis:

To study the relevance of the NSAID effect modification finding we report above, next, we conducted an analysis to determine the relationship between current NSAID use and serum estradiol levels. The results of this analysis resulted in a manuscript where the ongoing DOD funding is acknowledged for partial support. An account of this analysis is summarized below.

The manuscript, published in *Cancer Epidemiology Biomarkers and Prevention*, is included in the **Appendix** of this report.

Data were used from 260 cancer-free postmenopausal controls enrolled in the MAMS. Information on current NSAID use (aspirin, COX-2 inhibitors and other NSAIDs combined) was collected using a questionnaire at study enrollment. The primary exposure variable "current NSAID use" was based on a listing of current medications as reported by the participant on a self-administered questionnaire on the day of blood draw. Participants who listed current aspirin, COX-2 selective inhibitor, or other non-aspirin NSAID use on the questionnaire were considered "current NSAID users." Participants who did not report using any of these medications were considered "current NSAID non-users." Two additional NSAID exposure variables were considered, a secondary exposure variable and a NSAID variable constructed from the primary and secondary variables. The secondary NSAID exposure variable, which we called past 48 hour NSAID use, was from the participant's verbal response to the question at blood draw, "Have you taken any aspirin or anti-inflammatory drug in the past 48 hours?" The secondary exposure variable was used in conjunction with the primary NSAID exposure variable, to construct a third variable labeled "consistent NSAID use." Consistent NSAID users reported NSAID use on the questionnaire and verbally reported NSAID use. Consistent NSAID non-users did not report NSAID use for either measure. This variable was created as an attempt to reduce potential NSAID use/non-use misclassification. Serum estradiol was quantified by indirect radioimmunoassay at the Royal Marsden Hospital in London. General linear models were used to evaluate the association between NSAID use and serum estradiol, controlling for the effects of confounding variables. Estradiol levels were logarithmically transformed and geometric means are presented. A p-value of less than 0.05 was considered statistically significant. After adjustment for age and BMI, current NSAID use was significantly and inversely associated with serum estradiol concentrations (user=17.8 pmol/L vs. nonuser=21.3 pmol/L; p=0.03). The age-and BMI-adjusted association between use of the secondary NSAID exposure variable (aspirin or anti-inflammatory agent in the past 48 hours) and estradiol was suggestive of an inverse effect, but this finding was not statistically significant (user=18.5 pmol/L vs. non-user= 20.9 pmol/L; p=0.14). The strongest association was noted when comparing consistent NSAID users to consistent NSAID nonusers (user=17.5 pmol/L vs. non-user=21.5 pmol/L; p=0.03). Further adjustment for race, alcohol intake and years menopausal only slightly increased the strength of association observed in the age- and BMI- adjusted analyses (**Figure 2**).

In summary, we found that NSAID use was associated with lower serum estradiol levels among postmenopausal women not using exogenous hormones. The inverse relationship between NSAID use and estradiol was consistent regardless of how NSAID use was assessed.
Possibly through the inhibition of PGE2 synthesis and consequently on aromatase activity, NSAID use may lower serum estradiol levels and thereby reduce postmenopausal breast cancer risk. Thus, these results provide additional support to the mechanism proposed by which the n-6 and n-3 fatty acids are related to breast cancer.

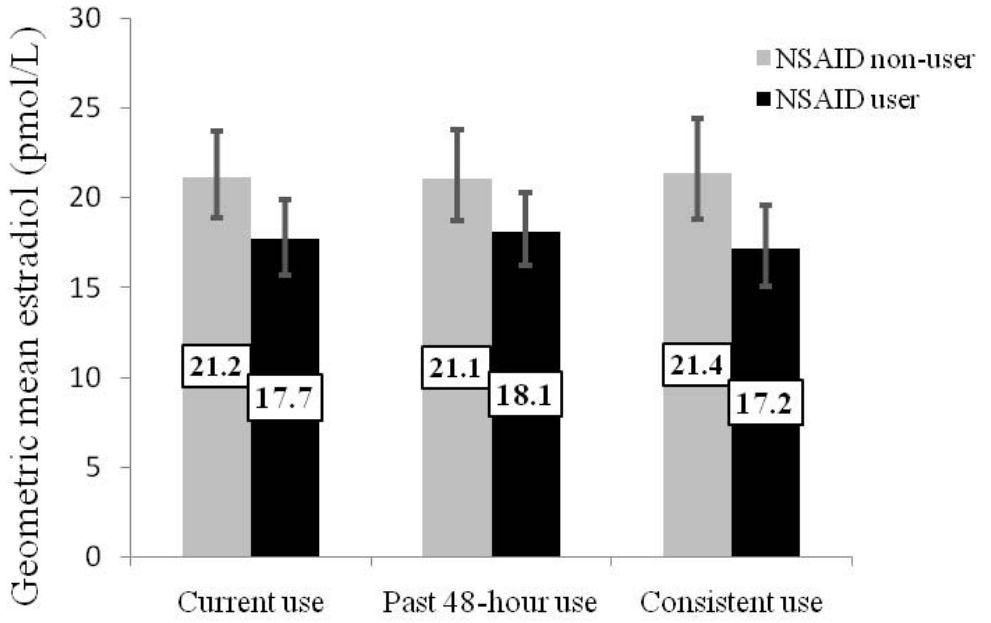

Figure 2. Multi-variable adjusted geometric mean serum estradiol concentration according to NSAID use

Omega-6 and omega-3 polyunsaturated fatty acids and breast density analysis:

Participants were only selected for this ancillary study if they met the following entry criteria: postmenopausal; no use of hormone therapy (HT) within 3 months of study enrollment; and not using vaginal estrogen creams, oral contraceptives, corticosteroids or SERMs on the day of blood sampling. Additionally, because MAMS utilized routine mammograms, the timing of the film does not coincide with the timing of the baseline blood draw, therefore participants whose time between film date and blood draw was greater than 120 days (~4 months), the lifetime of a red blood cell, were also excluded. Therefore 248 participants were included in this analysis. Mammographic breast density, dense area of the breast and nondense area of the breast were assessed by planimetry.

Unadjusted and adjusted Spearman rank correlation coefficients were determined and are presented in **Table 1**. No association was observed between any one of the erythrocyte fatty acid measures and percent breast density or dense breast area in either the unadjusted or adjusted analyses. Total n-3 fatty acids was inversely correlated with nondense area of the breast before adjustments were made for age and BMI. However, this association did not persist after adjusting for these variables. The total 6:3 PUFA ratio was positively and significantly correlated with nondense area of the breast, but again this finding diminished after correcting for covariates. Adjustment for additional confounding factors in a general linear model did not produce any statistically significant findings (data not shown). NSAID data was not available at the time of mammogram; hence the potential effect modification by NSAID use could not accurately be assessed.

In conclusion, in the present study we found no evidence of an association between n-6 or n-3 fatty acids and mammographic breast density in postmenopausal women. Thus, our results

suggest that if the n-6 and n-3 PUFAs affect the risk of breast cancer, it may not be through altering mammographic breast density. Manuscript preparation for this analysis is underway.

Table 1. Unadjusted and adjusted Spearman correlation coefficients between erythrocyte n-6 and n-3 polyunsaturated fatty acids and mammographic characteristics

Fatty acids (n=248)	Percent breast density	Dense breast area	Nondense breast area
Total n-6 PUFAs	-0.006 (0.93)	0.001 (0.99)	0.03 (0.69)
	0.02 (0.75)	-0.01 (0.87)	-0.04 (0.51)
Total n-3 PUFAs	0.10 (0.11)	0.01 (0.88)	-0.18 (0.004)
	0.02 (0.77)	0.02 (0.77)	-0.04 (0.51)
Total 6:3 PUFA ratio	-0.09 (0.15)	-0.01 (0.91)	0.16 (0.009)
	-0.01 (0.84)	-0.02 (0.78)	0.02 (0.65)

NOTE: First line is unadjusted estimates. Partial correlation estimates adjusted for age and BMI appear immediately below the unadjusted estimates. P-value between parentheses.

2.2 Training Activities

During the course of the second year, significant training accomplishments were made.

- Participated in a nutritional epidemiology course without benefit of a grade.
- Trained in the laboratory of Dr. Rhobert Evans (University of Pittsburgh). Observed gas-liquid chromatography, high performance liquid chromatography and enzyme-linked immunoassays.
- Published 2 manuscripts resulting from my training (training during months 0-12) on the PREFER study, a dietary intervention study.
    - Burke L, Warziski M, Styn M, Music E, **Hudson A**, Sereika S. A randomized clinical trial of a standard versus vegetarian diet for weight loss: the impact of treatment preference. Int J Obes. 2008; 32: 166-76.
    - Burke L, **Hudson A**, Styn M, Warziski M, Ulci O, Sereika S. Effects of a vegetarian diet and treatment preference on biological and dietary variables in overweight and obese adults: a randomized trial. Am J Clin Nutr. 2007; 86: 588-596).
- Attended the 2008 University of Pittsburgh Graduate School of Public Health's Career Day to develop and confirm a working relationship with businesses, agencies and institutions engaged in public health concerns, Pittsburgh, PA
- Attended numerous scientific presentations at the University of Pittsburgh, including topics such as:
    - "Culture in Epidemiology Research" Speaker: Stephen Thomas, PhD
    - "Lessons Learned from Two of the Longest and Largest Epidemiology Studies involving U.S. Minority Populations" Speaker: Barbara Howard, PhD
    - "Ethical Issues in International Research" Speaker: Clareann Bunker, PhD
- Attended in the University of Pittsburgh Survival Skills workshop entitled, "Writing Research Articles."

- Attended the Society for Epidemiologic Research (SER) Annual Meeting 2007 Boston, MA.
  - One abstract, on NSAID use and estradiol levels (reported above in section 2.1; **also see Appendix**) was presented in the form of a poster presentation.
  - One abstract, on breast density in the MAMS, was accepted and presented in the form of a poster presentation.
- One abstract, on NSAID use and postmenopausal estradiol levels in the MAMS (reported above in section 2.1), was accepted to the University of Pittsburgh Graduate School of Public Health's 10th Annual Dean's Day Competition. This presentation received 2nd place for best overall presentation.
- One abstract on the Gail Model, a breast cancer risk assessment tool, and sex steroid hormone levels was submitted and accepted to the American Society of Clinical Oncology Annual Meeting 2008.
- One abstract was submitted and accepted to the Breast Cancer Era of Hope 2008 meeting

3 Key Research Accomplishments

During the course of the second year (months 13-24) of funding we have shown that:

- Erythrocyte n-6 PUFAs and the total 6:3 PUFA ratio are positively related to serum estradiol levels, whereas n-3 PUFAs are negatively related to estradiol levels among NSAID non-users. Similar findings were not observed among NSAID users. The preliminary results of this analysis were reported at a scientific conference during months 1-12 and manuscript preparation is underway.
- NSAID users have lower circulating estradiol levels than NSAID non-users. This finding was presented at a scientific conference and published in a peer-reviewed journal.
- Analyses did not reveal an association between erythrocyte n-6 and n-3 PUFAs and mammographic breast density. This suggests that if the n-6 and n-3 fatty acids influence breast cancer development, it may not be through altering breast density.

4 Reportable Outcomes

Reportable outcomes for months 13-24 are as follows:

Manuscripts:

- **Hudson A**, Gierach G, Modugno F, Simpson J, Vogel V, Wilson J, Evans R, Weissfeld J. Nonsteroidal anti-inflammatory drug use and serum total estradiol in postmenopausal women. Cancer Epidemiol Biomarkers Prev March 2008;17(3).

Abstracts:

- Hudson A, Gierach G, Modugno F, Simpson J, Vogel V, Wilson J, Evans R, Weissfeld J. Anti-inflammatory drug use and serum estradiol in postmenopausal women. Society for Epidemiological Research Annual Meeting 2007. Boston, MA. June 2007. Abstract published in Amer J Epidemiol. 2007, 156 (11) #326-S.

Presentations:

- **Hudson A**, Gierach G, Modugno F, Simpson J, Vogel V, Wilson J, Evans R, Weissfeld J. Anti-inflammatory drug use and serum estradiol in postmenopausal

women. University of Pittsburgh Graduate School of Public Health Dean's Day, Pittsburgh, Pennsylvania, March 2008.

5 Conclusion

During the second year of funding, we have made significant progress towards our research goals. In summary, we observed a positive relationship between n-6 fatty acids and the 6:3 PUFA ratio and serum estradiol concentrations and inverse associations between both the n-3 fatty acids and NSAID use and serum estradiol. Interestingly, the significant associations observed between the erythrocyte n-6 and n-3 fatty acid measures and estradiol concentrations were observed among NSAID nonusers, but not among current NSAID users. Contrary to our hypotheses, we did not observe an association between any one of the fatty acid measures with mammographic density. Therefore, if the n-6 and n-3 fatty acids influence breast cancer risk, it may not be through affecting breast density. To our knowledge, none of the aforementioned relationships have previously been explored.

"So What"

We observed positive relationships between erythrocyte n-6 fatty acids and the 6:3 PUFA ratio and inverse relationships between n-3 fatty acids and NSAID use with circulating estradiol concentrations. The relationship between circulating levels of estradiol and breast cancer risk is greatly documented; hence, the identification of modifiable factors capable of altering this well-established breast cancer risk factor could have a substantial impact on public health as it could aid in the development of chemopreventive guidelines.

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7 Appendices

1. Curriculum Vitae for Ms. Alana Hudson (pages 12-13)
2. **Hudson A**, Modugno F, Wilson J, Evans R, Gierach G, Vogel V, Simpson J, Weissfeld J. Erythrocyte omega-6 and omega-3 fatty acids and postmenopausal serum total estradiol. (anticipated manuscript; under co-author review) (pages 14-36)
3. **Hudson A**, Gierach G, Modugno F, Simpson J, Vogel V, Wilson J, Evans R, Weissfeld J. Anti-inflammatory drug use and serum estradiol in postmenopausal women. Society of Epidemiological Research Annual Meeting 2007. Boston, MA. June, 2007. Abstract. (page 37)
4. Hudson A, Gierach G, Modugno F, Simpson J, Wilson J, Evans R, Vogel V, Weissfeld J. NSAID use and serum total estradiol in postmenopausal women. Cancer Epidemiol Biomarkers Prev. March 2008;17(3): 680-87. (pages 38-45)

Alana G. Hudson, PhD Candidate

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PROFESSIONAL EXPERIENCE

2006-Present **Department of Defense Predoctoral Fellow**, Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania
Responsibilities include conducting data analyses and dissertation/manuscript preparation for the Mammograms and Masses Study (MAMS), a case-control study on estrogen metabolites, mammographic breast density and breast cancer risk.

2004-2006 **Graduate Student Researcher,** Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania
Primary responsibilities included writing study proposals and conducting data analyses for the MAMS.

2003-2004 **Research Assistant,** Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania
Responsibilities included collecting clinical data, interviewing study participants, and developing study protocols and research tools for MAMS.

EDUCATION

2003-Present **University of Pittsburgh,** Pittsburgh, Pennsylvania
Doctor of Philosophy in Epidemiology

1998-2002 **West Virginia University,** Morgantown, West Virginia
Bachelor of Science in Human Nutrition

1998-2002 **West Virginia University,** Morgantown, West Virginia
Bachelor of Science in Animal Science

TRAINING

2004 **Ovarian Cancer Speaker's Bureau Training**
National Ovarian Cancer Coalition (NOCC), Pittsburgh, Pennsylvania

SCHOLARSHIPS

2004 Public Health Dean's Scholarship, University of Pittsburgh, for academic merit, community service, and financial need
1998-2002 Mountaineer Scholarship, West Virginia University, for academic achievement
1998 Berry Scholarship, West Virginia University, for scholarly performance

HONORS AND AWARDS

2008 Received second place for best overall presentation, 10th Annual University of Pittsburgh Graduate School of Public Health's Dean's Day Competition
2007 American Association for Cancer Research (AACR)-AFLAC, Incorporated Scholar-in-Training Award to present research at the 2007 AACR annual meeting
2007 Received second place for best overall presentation, 9th Annual University of Pittsburgh Graduate School of Public Health's Dean's Day Competition
2002 Graduated *cum laude*
1998-2002 Elected to National Society of Collegiate Scholars, Golden Key National Honor Society, Phi Upsilon Omicron Honor Society and Gamma Beta Phi Society

Alana G. Hudson, PhD Candidate

FUNDING

2006-Present Principal Investigator: "The 6:3 polyunsaturated fatty acid ratio and intermediate markers of breast cancer." Funded by the U.S. Army Medical Research and Material Command (USAMRMC), Department of Defense Breast Cancer Research Program, Predoctoral Award. $89,996

PUBLICATIONS

Hudson A, Gierach G, Modugno F, Simpson J, Wilson J, Evans R, Vogel V, Weissfeld J. NSAID use and serum total estradiol in postmenopausal women. *Cancer Epidemiol Biomarkers Prev.* March 2008;17(3): 680-87.

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Hudson A, Modugno F, Wilson J, Evans R, Vogel V, Gierach G, Simpson J, Weissfeld J. Omega-6 and omega-3 fatty acids in erythrocytes and serum total estradiol concentrations among postmenopausal women (under co-author review).

Erythrocyte omega-6 and omega-3 fatty acids and postmenopausal serum total estradiol

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Running Title: Erythrocyte fatty acids and estradiol

Keywords:

Support: This work was supported in part by National Institutes of Health grants R25-CA57703, K07-CA80668, R21-CA95113, and P20 CA103730-02; by Department of Defense grants DAMD17-02-1-0553 and W81XWH-06-1-0532 and by PA Department of Health grant P2777693. Additional support was provided by funds received from the NIH/NCRR/GCRC Grant MO1-RR000056.

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ABSTRACT

Elevated intake of omega-6 (n-6) polyunsaturated fatty acids (PUFAs) may promote breast cancer, whereas omega-3 (n-3) consumption may inhibit the growth of this disease. The mechanism by which these fatty acids impact breast cancer development is unknown; however, experimental evidence indicates that these two families of fatty acids may influence risk by impacting eicosanoid synthesis. Specifically, when n-3 PUFAs displace n-6 PUFAs, prostaglandin E2 production (PGE2) is reduced, resulting in decreased aromatase activity and ultimately suppression of estrogen synthesis. Thus, in this cross-sectional analysis, we sought to determine whether n-6 and n-3 fatty acids in erythrocytes, expressed as a percentage of total fatty acids, were associated with postmenopausal serum total estradiol concentrations. Because NSAIDs also inhibit PGE2 formation, separate analyses were performed for participants using and not using NSAIDs. Among women not using NSAIDs (n=135), multivariate adjusted estimates revealed that mean estradiol concentrations decreased with increasing tertile of total erythrocyte n-3 fatty acids (24.3 pmol/L vs 18.4 pmol/L; $p<0.05$) and increased with increasing tertile of total n-6 fatty acids (16.0 pmol/L vs. 21.8 pmol/L; $p=0.02$), the total n-6:n-3 ratio (17.6 pmol/L vs. 22.9 pmol/L; $p=0.06$) and the ratio of n-6 arachidonic acid to n-3 eicosapentaenoic acid+docosahexaenoic acid (17.6 pmol/L vs. 24.9 pmol/L; $p<0.01$). Among NSAID users (n=118), mean estradiol was greatest among women in the highest tertile of the n-6 linoleic acid to n-3 alpha-linolenic acid (ALA) ratio as compared to the lowest tertile (21.1 pmol/L vs. 14.2 pmol/L; $p=0.01$). This finding was primarily due to the inverse association noted between ALA and estradiol. NSAID users in the highest tertile of ALA had a lower mean estradiol concentration than participants in the lowest tertile of ALA (15.2 pmol/L vs. 20.8 pmol/L; $p=0.05$). No other significant differences were noted among current NSAID users. Because circulating postmenopausal estradiol concentrations are causally related to breast carcinogenesis, these findings provide a mechanism through which the n-6 and n-3 fatty acids may alter breast cancer risk.

INTRODUCTION

Breast cancer is a common form of cancer among women worldwide and despite substantial advances in the treatment of this disease breast cancer remains a leading cause of death among women. Several lines of evidence implicate that dietary intake may influence breast cancer, and for many years it has been postulated that excessive dietary fat consumption may play a role in the etiology of this disease [1]. However, no clear consensus on this topic has been established. One theory that addresses this debate is that a relationship may not exist between breast cancer risk and total fat intake, but an association may exist between breast cancer and the type of fat consumed. Considerable interest has focused on the association between the omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) and breast cancer risk, largely stemming from analysis of international data [2, 3].

The majority of PUFAs in the United States diet, where breast cancer rates are among the highest in the world, consist of n-6 PUFAs found in abundance in corn and vegetable oils [4, 5]. Populations such as the Greenland Eskimos [6], the Alaskan Natives [7] and the Japanese [8] have high fish consumption, hence high n-3 intake. These populations also have substantially lower rates of breast cancer despite their overall high fat consumption [6, 9, 10]. Therefore, the higher rates of breast cancer observed in the United States may be explained, in part, by the elevated intake of n-6 and/or insufficient intake of n-3 PUFAs. This hypothesis is supported by some experimental [11-20] and epidemiological data [21-24].

Perhaps the most acknowledged mechanistic pathway through which the n-6 and n-3 fatty acids may influence breast cancer risk is via eicosanoid biosynthesis. Arachidonic acid (AA; 20:4n-6), which can be ingested or formed endogenously by desaturation and elongation of linoleic acid (LA; 18:2n-6), serves as the substrate for cyclooxygenase (COX) mediated prostaglandin E2 (PGE2) production. PGE2 is an inflammatory eicosanoid that is upregulated in breast tumors [25] and is a potent inducer of aromatase activity [26], the key enzyme in postmenopausal estrogen synthesis. In contrast, prostaglandin E3 (PGE3), an anti-inflammatory eicosanoid, is derived from the metabolism of eicosapentaenoic acid (EPA; 20:5n3). EPA can be consumed, formed from the essential n-3 fatty acid alpha-linolenic acid (ALA; 18:3n-3), or formed from retroconversion of docosahexaenoic acid (DHA; 22:6n-3). Unlike PGE2, PGE3 has not been documented to upregulate aromatase and is signficiantly less mitogenic [27].

The n-6 and n-3 PUFAs compete with each other for enzymes at multiple levels; therefore, increasing n-3 consumption ultimately suppresses the production of PGE2 [27, 28]. This suggests that reducing n-6 or increasing n-3 intake, which results in lowered PGE2 formation [27-29], may result in lower circulating estradiol levels. Indeed, nonsteroidal anti-inflammatory drugs (NSAIDs), which also inhibit formation of PGE2, are associated with reduced estradiol production in breast tumor cells [30] and lower serum estradiol concentrations in postmenopausal women [31].

Little is known about the relationship between n-6 and n-3 PUFAs and circulating postmenopausal estrogen levels, likely a result of the methodological issues with the estimation of individual fatty acids from self-reported dietary instruments. Utilizing fatty acids in erythrocytes allows for individual fatty acid assessment, provides an objective measurement, and reflects recent dietary intake of the essential n-6 and n-3 fatty acids. Therefore, we evaluated the association between dietary habits of the n-6 and n-3 PUFAs, as inferred from erythrocyte fatty acid composition, and serum total estradiol concentration in postmenopausal women.

MATERIALS AND METHODS

Study Population

Details of the Mammograms and Masses Study (MAMS) have been described previously [32]. The MAMS is a case-control study of estrogen metabolites, mammographic breast density and breast cancer risk. A total of 869 cancer-free women and 264 recently diagnosed breast cancer cases were recruited into the MAMS through the Magee Womens Hospital Mammographic Screening and Diagnostic Imaging Program in the greater Pittsburgh area (Pennsylvania, USA) in 2001-2005. The participants were all women aged 18 years or older and who reported no previous personal history of cancer, with the exception of nonmelanoma skin cancer. Participants in the MAMS include: 1) newly diagnosed breast cancer cases who were recruited from the Magee-Womens Surgical Clinic (n=264); 2) women who were undergoing outpatient needle breast biopsy through the Breast Biopsy Service at Magee-Womens Hospital, but were not subsequently diagnosed with breast cancer (n=313); 3) cancer-free women who received screening mammography through Magee-Womens Hospital or through Magee Womancare Centers (n=538) and; 4) an additional 18 participants whose blood was dedicated

solely to an ancillary study of intra-individual cytokine and hormone concentration reproducibility. To increase recruitment of the "healthy" control group, study flyers were attached to screened negative mammogram reports mailed to patients from 2003-2005. The study was approved by the Institutional Review Board of the University of Pittsburgh and all participants provided written informed consent.

Subsample Selection

Inclusion criteria for entry into this ancillary study were as follows: 1) controls recruited only via study flyers through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers (n=453), as information on these participants was gathered on the day blood was drawn; 2) postmenopausal (having had no menstrual bleeding during prior year or having undergone a bilateral oophorectomy); 3) not using hormone therapy (HT) within three months of enrollment; and 4) not using vaginal estrogen creams, oral contraceptives, selective estrogen receptor modulators (SERMs) or corticosteroids at study enrollment. A total of 270 women met the inclusion criteria for the present analyses. Of those who were excluded, 98 were premenopausal, 84 were using exogenous hormones, SERMs, or corticosteroids, and 1 participant was later diagnosed with breast cancer.

Covariate Information

We used a standardized, self-administered questionnaire to gather participants' exposure information at study enrollment. Information on demographic characteristics, current use of medication and supplements, reproductive history, family medical history, past exogenous hormone use, smoking status, and alcohol consumption was obtained. Participants were asked to report all prescribed and over-the-counter medications that were currently being used on the questionnaire. Women who listed using aspirin, COX-2 inhibitors, or other non-aspirin NSAIDs were considered "current NSAID users." Participants who did not list using a NSAID were considered "current NSAID non-users." Because acetaminophen is generally reported to be a weak inhibitor of the COX -1/COX-2 enzymes [33], we classified acetaminophen users as non-users of NSAIDs unless they also reported taking a NSAID. Regular alcohol use (g/day) in the past year was calculated as previously described [34]. Age of menopause was defined according to the methods reported by the Women's Health Initiative [35], where age at menopause

corresponded to the age of a woman's last natural menstrual bleeding, bilateral oophorectomy, or age a woman began using HT. For a hysterectomized woman without a bilateral oophorectomy, age at menopause was the earliest age at which she began using HT or first had menopausal symptoms. If neither occurred and her age at hysterectomy was 50 years or older, then age at menopause was her age at hysterectomy. Age at menopause could not be determined in 7 participants. Years since menopause were calculated by subtracting a woman's age at menopause from her age at study enrollment. The questionnaire was reviewed for completeness by a trained research nurse.

Clinical Measures

After participants removed shoes and heavy clothing, height and weight were measured by the study nurse. Weight was measured at a standing position to the nearest 0.1 kg using a standard balance beam; standing height was measured at full inspiration to the nearest 0.1 cm. Measurements were taken twice and were repeated if the first two measurements differed by more than 0.5 cm or 0.5 kg. The mean of the measurements was used to derive final heights and weights. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

After anthropometric assessment, a 40 ml non-fasting blood sample was collected by the study nurse. Samples were processed on site according to a standardized protocol. After processing, the samples were separated into red blood cell, serum, plasma and buffy coat and stored at or below -70°C or below until assayed.

Measurement of Fatty Acids

Erythrocyte fatty acid concentrations were identified using gas-liquid chromatography. Samples were analyzed at the University of Pittsburgh's Heinz Laboratory. Total lipids (500µl of packed red blood cells) were extracted according to the general technique of Bligh and Dyer [36]. Briefly, the samples were homogenized in 4 ml of methanol, 2 ml of chloroform and 1.1 ml of water. Two ml of chloroform and 2 ml of water were added to the samples after 15 min. The tubes were then centrifuged at 1200 g for 30 min at 16°C and the upper phase discarded. The lower phase was dried under nitrogen and resuspended in 1.5 ml 14% boron trifluoride methanol. The samples were heated at 90°C for 40 min and after cooling extracted with 4.0 ml pentane and 1.5 ml water. The mixtures were vortexed and the organic phase recovered [37].

The extracts were dried under nitrogen, resuspended in 50 µl heptane and 2 ml injected into a capillary column (SP-2380, 105 m x 53 mm ID, 0.20 um film thickness). Gas chromatographic analyses were carried out on a Perkin Elmer Clarus 500 equipped with a flame ionization detector. Operating conditions were as follows: the oven temperatures were 140°C for 35 min; 8°C/min to 220°C, held for 12 min; injector and detector temperatures were both at 260°C; and helium, the carrier gas, was at 15 psi. Identification of fatty acids was by comparison of retention times with those of authentic standards (Sigma). A random subset of 27 samples was analyzed for reproducibility; laboratory personnel were blinded to duplicate samples and subject identification. The inter-assay coefficients of variation (CV) for the fatty acid measures reported ranged between 1.7-15.2%. CVs's were 4.6% for LA, 3.4% for AA, and 1.7% for total n-6 fatty acids. CV's were higher for the n-3 fatty acids, with CV's of 15.2% for ALA, 5.3% for EPA, 7.5% for DHA and 5.3% for total n-3 fatty acids. The CVs for the total n-6:n-3, LA:ALA, and AA:EPA+DHA ratios were 5.2%, 11.1%, and 5.7% respectively. The individual and total n-6 and n-3 fatty acids are expressed as a percentage by weight of the total erythrocyte fatty acid content.

Measurement of Total Estradiol

Serum total estradiol was measured by radioimmunoassay (RIA) after diethyl ether extraction using a highly specific rabbit antiserum raised against an E2-6-carboxymethyloxime-BSA conjugate (EIR, Wurenlingen, Switzerland) and Third Generation Estradiol [I125] reagent DSL 39120 (Diagnostic Systems Laboratories Inc., Texas USA). Assays were conducted at the Royal Marsden Hospital in England [38]. The lower detection limit of the assay was 3pmol/L by calculation from the 95% confidence limits of the zero standard. Twenty-seven replicate quality control samples were analyzed to assess reproducibility; the calculated CV between duplicates for estradiol was 14.5%. Laboratory personnel were blinded to quality control status.

Statistical Analyses

Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Inc., Cary, North Carolina). To improve normality for statistical tests, a log transformation was applied to serum total estradiol concentrations. One participant was excluded from analyses because total estradiol concentrations were deemed unreliable by the laboratory. An additional 9

participants with estradiol values greater than 150 pmol/L were removed from analyses because such high levels likely indicated the participants were not postmenopausal or incorrectly reported current hormone use. Analyses were repeated with extreme data points included, and because findings did not change substantially the 9 participants were not included in the final report. The final sample included 260 women.

Descriptive results for continuous variables are expressed as means and standard deviations (SD). Categorical variables are reported as frequencies and percentages (%). Correlation relationships between n-6 and n-3 fatty acid measures and serum estradiol were examined with Spearman's correlation coefficients, with no adjustments and controlling for the effects of age and BMI.

Multivariate associations for serum total estradiol according to tertile of fatty acid were assessed by analysis of covariance (ANCOVA) using the general linear models (GLM) procedure of SAS (PROC GLM). Adjusted geometric mean estradiol and 95% confidence intervals (CI) were calculated using least squares means, controlling for the effects of age (continuous), BMI (continuous), years menopausal (continuous), regular alcohol consumption in the past year (0g/day, <12g/day, ≥12g/day, entered as a dummy variable), and current smoking status (nonsmoker vs. smoker). The comparisions were adjusted for these variables as they were strongly related to the majority of erythrocyte fatty acid measures, and with the exception of smoking status, were also related to serum estradiol levels. The geometric mean concentrations of estradiol were calculated by taking the anti-log of the least squares means after adjustment. NSAIDs reduce PGE2 synthesis and hence aromatase activation, thus, intake of the n-6 and n-3 PUFAs may have less of an effect on estradiol levels among NSAID users. Therefore, we performed analyses stratified by NSAID use to assess possible effect modification by this variable. To formally test whether the effects of fatty acids were altered by current NSAID use, an interaction term between fatty acid (tertile) and NSAID use was entered into the unstratified multivariate model. Tests of linear trends were performed across fatty acid measures by modeling tertiles as consecutive integers (continuous variable).

The assumptions of the models were checked by residual analysis. Plots of the residuals versus the predicted values were examined to check for heteroscedasticity. The normal probability plot of the residuals was examined to assess the normality of the error terms. Model assumptions of normality and homogeneity of variance were met for all models presented. Tests

of statistical significance were two-tailed and given the exploratory nature of this work, we reported our results at the $p<0.05$ significance level, rather than correct for multiple comparisons. Analyses were repeated excluding participants (n=13) reporting fatty acid supplementation at blood draw, but results did not differ substantially and are therefore not presented.

RESULTS

The characteristics of the study population are shown in **Table 1**. The mean (SD) age was 62.8 (8.4) years and the mean (SD) BMI was 28.5 (6.0) kg/m^2. Only 6.9% of the women were non-white and 5.8% current smokers. Close to half (47.7%) of the population indicated current use of a NSAID (aspirin, non-aspirin, and/or COX-2 inhibitor) at study enrollment. The geometric mean serum estradiol concentration for the study population was 19.5 pmol/L, with levels ranging from 3.3-140.0 pmol/L.

On average, the proportion of total n-6 fatty acids was higher than the proportion of total n-3 fatty acids in erythrocytes (**Table 2**). The average ratio of mean total n-6 fatty acids to mean total n-3 fatty acids was 5.2. N-6 AA and LA were the most abundant fatty acids, with AA composing 16.0% and LA 15.8% of total fatty acids. Of the n-3 fatty acids, DHA accounted for the greatest percentage (4.5%) of total fatty acids.

Table 3 presents the unadjusted and adjusted correlations between fatty acid measures and serum total estradiol for the entire study population. Analyses revealed distinct differences in the relationships of the erythrocyte n-6 and n-3 fatty acids with serum estradiol. Statistically significant positive correlations were observed for both total n-6 fatty acids ($r=0.15$, $p=0.02$) and AA ($r=0.13$, $p=0.04$) with estradiol. A nonsignificant inverse association was observed for n-6 LA ($r=-0.08$, $p=0.21$). Erythrocyte total n-3 fatty acids and all individual n-3 fatty acids were inversely related with serum total estradiol. Correlation coefficients ranged from -0.17 to –0.24 and were statistically significant ($p<0.05$) for all n-3 fatty acid measures. The strongest inverse correlation was found for EPA ($r = -0.24$, $p<0.0001$), followed closely by total n-3 fatty acids ($r=-0.22$, $p=0.0003$). Highly significant positive correlations between serum estradiol and the three 6:3 PUFA ratios (total n-6:n-3, LA:ALA, and AA:EPA+DHA) were found. Spearman correlation coefficients ranged from 0.18 ($p < 0.004$) to 0.23 ($p < 0.0002$). Adjustment for age and BMI attenuated the findings.

Table 4 shows the estimated geometric mean of serum estradiol concentration across tertile of erythrocyte fatty acid. After adjustment for age, BMI, years menopausal, alcohol intake, current smoking, and current NSAID use, no individual fatty acid was significantly related to estradiol concentration. However, a significant trend of increasing estradiol concentration with tertile of LA:ALA (p trend=0.03) was found. The adjusted total estradiol concentration was approximately 20.5% higher among participants in the highest tertile of LA:ALA as compared to those in the lowest tertile. Although a higher mean estradiol concentration was observed in the highest tertile as compared to the lowest tertile of all other 6:3 ratios (total n-6:n-3, AA:EPA, and AA:EPA+DHA), the findings were not statistically significant. A suggestive inverse trend for ALA tertiles (p trend =0.09) was noted.

Estradiol and fatty acids among NSAID non-users

Because NSAIDs inhibit the COX/PGE2/aromatase pathway, we next explored the associations between fatty acids and estradiol by NSAID use. **Table 4** shows the estimated geometric mean estradiol concentrations across tertile of fatty acid stratified by NSAID use. Among non-users of NSAIDs, multivariate adjusted analyses revealed several significant or borderline significant associations between fatty acid measures and estradiol. Total n-6 fatty acids was positively and significantly related to serum estradiol (p trend=0.02). The adjusted geometric mean estradiol levels also rose with increasing tertile of n-6 AA among NSAID non-users, with mean estradiol levels 24.2% higher in the topmost tertile as compared to the lowest tertile (p trend=0.09). No association was observed between LA and estradiol (p trend=0.97). On the contrary, geometric mean serum estradiol levels decreased with increasing tertile of total n-3 fatty acids (p=0.05), with mean estradiol levels 24.3% lower in the highest tertile as compared to the lowest. Mean estradiol levels were also lower in the highest tertile of all individual n-3 fatty acids (ALA, EPA, and DHA) as compared to the lowest tertile; however, the p for trend did not reach statistical significance for any of these measures. The total n-6:n-3 ratio was positively linked to estradiol and the finding approached statistical significance (p trend=0.06), with 30.1% greater mean estradiol levels in the upper tertile as compared to the lower tertile. A positive relationship was observed between the AA:EPA+DHA ratio and estradiol, with mean estradiol concentrations 40.7% higher in the highest as compared to the lowest tertile (p=0.01). The geometric mean estradiol level was also higher in the LA:ALA

ratio's uppermost tertile as compared to the lowest tertile; however, this trend was not statistically significant.

Estradiol and fatty acids among NSAID users

Among women reporting current NSAID use, a positive association between the LA:ALA ratio and estradiol was noted, with mean estradiol in the highest tertile 48.6% higher than the mean estradiol of the lowest tertile (p trend=0.01). This observation was largely attributable to n-3 ALA, which was inversely related to estradiol in NSAID users (p trend=0.05). Estradiol was 26.9% lower in the highest tertile of ALA as compared to the lowest tertile. No other fatty acid measure was related to serum total estradiol levels within the NSAID user stratam.

Effect modification by NSAID use was formally tested by including interaction terms in the GLMs. Despite substantial differences in the relationships between fatty acids and estradiol between NSAID users and non-users, the only signficant interaction was between NSAID use and total n-6 fatty acids with respect to circulating estradiol ($p<0.02$). The interaction between NSAID use and the AA:EPA+DHA ratio was suggestive (p=0.12). However, this study had limited power to detect interaction effects.

CONCLUSIONS

In this cross-sectional investigation, we examined the relationships between erythrocyte n-6 and n-3 fatty acids and postmenopausal serum total estradiol concentrations. In a population of women not reporting current NSAID use, we found a positive association between total n-6 fatty acids and estradiol and an inverse association between total n-3 fatty acids and estradiol. We further observed positive relationships between the AA:EPA+DHA ratio and the total n-6:n-3 ratio with serum estradiol. As high postmenopausal circulating estradiol concentrations are related to increased breast cancer risk, these findings are consistent with the hypothesis that n-6 fatty acids may increase the risk of breast cancer and n-3 fatty acids may protect against this disease. Similar associations were not noted among women who reported current NSAID use. Although, none of the significant findings observed among non-users of NSAIDs were found among current NSAID users, a significant positive relationship was noted between the LA:ALA

ratio and estradiol levels. This relationship was largely attributable to the inverse association between ALA and estradiol.

A potential explanation for the null finding between n-6 fatty acids and estradiol among NSAID users is that since NSAIDs inhibit PGE2 formation, limiting the amount of substrate available (n-6 fatty acid AA) for PGE2 synthesis is not of biological importance. In addition, a relationship between total n-3 fatty acid measures and estradiol might not have been observed among NSAID users, because both n-3 consumption and NSAID use reduce PGE2 production, and therefore exposure to both anti-inflammatory agents might not offer additional benefit. The strong inverse relationship noted between ALA and estradiol in conjunction with the speculations offered above, may be suggestive that an additional pathway is involved other than ALAs ability to compete for COX enzymes (i.e. through elongation to EPA) among NSAID users. Possible mechanisms of action include ALA's ability to reduce TNF-alpha and IL-6 [39], which have also been shown to stimulate aromatase activity [40]. Although this finding may be biologically plausible, we also must acknowledge the potential role chance plays when multiple comparisons are made.

Although the majority of interactions between fatty acid measures and NSAID use were not statistically significant, the effect sizes suggest that there are differences in the strengths of the relationships between NSAID non-users and users. Thus the lack of significance of the interaction terms may be attributable to the low statistical power of our study. However, it should also be acknowledged that given the nonsignificant interaction terms, the effect modification noted may be a result of chance findings. Nonetheless, given the biological plausibility of an interaction and because no study has previously reported on these associations, we chose to present the data stratified by NSAID use.

We are unaware of any study reporting weaker effects of the n-6 and n-3 fatty acids on breast cancer risk among NSAID users; however, long chain n-3 PUFA levels in blood were associated with decreased colorectal cancer risk among aspirin non-users, but not among aspirin users [41]. Futher a statistically significant interaction between dietary fat intake and NSAID use ($p=0.007$) has been noted. Among non-users of NSAIDs, decreasing fat intake was inversely related to recurrence of adenomous polyps [42]. A similar finding was also noted in relation to squamous cell carcinoma of the skin. Erythrocyte levels of n-6 AA were significantly greater

among cases than controls and this relationship was more apparent among NSAID non-users [43].

To our knowledge this is the first study to report on the relationship between the essential fatty acids found in biospecimens and endogenous estradiol levels. Further, there is a paucity of data on the impact of n-6 and n-3 fatty acids, as measured via self-report, on concentrations of postmenopausal estradiol levels. Consistent with our findings, estradiol levels have been found to be significantly inversely related to n-3 fat from fish [44]. We are not aware of any epidemiological investigation that assessed the relationship between individual or total n-6 fatty acids or n-6:n-3 ratios and postmenopausal estradiol levels.

A key limitation of this study is its cross-sectional nature, which does not allow causal inference. Hormone and erythrocyte fatty acid concentrations were measured once and a single measure may be inadequate to some degree because of variability within individuals over time; for that reason, assaying multiple samples over time might better characterize levels in these women. However, use of a single measurement of erythrocyte membranes is capable of reflecting recent n-6 and n-3 fatty acid intake [45]. We cannot rule out that perhaps n-6 and n-3 levels are simply markers of poor or healthy lifestyles. While we adjusted for BMI, alcohol intake and smoking in multivariate analyses, relations between the fatty acids and estradiol levels could be due to residual confounding by unmeasured lifestyle characteristics rather than real dietary effects. Additionally, the MAMS participants included in this analysis are a relatively homogenous sample as all are postmenopausal, not using hormone therapy, and predominantly white, thus the study results may have limited generalizability. In spite of these limitations, this study is unique in that we believe no other epidemiological study has assessed the relationship between circulating fatty acids and serum estradiol levels. Additional study strengths include the use of an objective measure of dietary fat intake and standardized assessment of participant characteristics.

In summary, this study provides modest evidence supporting a positive association between n-6 PUFAs and a negative association between n-3 PUFAs and serum estradiol levels. However, given the cross-sectional design of the study, the observed relationships should be viewed as hypothesis generating and interpreted with caution. The answers to the role of the essential n-6 and n-3 PUFAs in breast cancer development are not definitive, the data being too insufficient to be convincing. Because of limitations in current research, chemoprotective dietary

recommendations for women cannot be issued. Given this study's findings and the limitations listed above, prospective studies assessing the relationship between the n-6 and n-3 PUFAs and circulating estrogens using validated dietary assessment instruments along with repeated blood sampling for fatty acid and estradiol analysis are warranted.

Table 1. Distribution of selected characteristics among postmenopausal women in the Mammograms and Masses Study

Continuous variables	mean	SD
Age at blood draw (years)	62.8	8.4
BMI (kg/m^2)	28.5	6.0
Age at menopause (years)*	48.7	5.1
Years menopausal*	14.1	10.0
Categorical variables	**n**	**%**
Race		
White	242	93.1
Non-white	18	6.9
Surgical menopause *		
No	229	88.4
Yes	30	11.6
Prior hormone therapy use		
No	100	38.5
Yes	160	61.5
Regular alcohol intake in past year		
None	188	72.3
< 12 g/day	46	17.7
≥ 12 g/day	26	10.0
Current Smoker		
No	245	94.2
Yes	15	5.8
Current NSAID use		
No	136	52.3
Yes	124	47.7

NOTE: BMI, body mass index; NSAID, nonsteroidal anti-inflammatory drug
* Mean and prevalence estimates were determined on nonmissing data; missing n=7 for age at menopause, n=7 for years menopausal, and n=1 for surgical menopause

Table 2. Mean fatty acid composition in erythrocytes

Fatty Acids (wt. %)	mean (SD)
Total n-6 PUFA*	38.3 (2.6)
18:2n-6 (LA)	15.8 (2.4)
20:4n-6 (AA)	16.0 (2.0)
Total n-3 PUFA †	7.9 (2.0)
18:3n-3 (ALA)	0.2 (0.1)
20:5n-3 (EPA)	0.9 (0.4)
22:6n-3 (DHA)	4.5 (1.5)
6:3 Ratios	
Total n-6:n-3	5.2 (1.5)
LA:ALA	72.7 (19.3)
AA:EPA+DHA	3.3 (1.2)

NOTE: N=260. Data are expressed as mean (SD). Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid.

*18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

†18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

Table 3. Spearman rank order correlation coefficients between n-6 and n-3 fatty acids in erythrocytes and serum estradiol concentrations

Fatty Acids (wt. %)	r*	p	r†	p
n-6 PUFAs				
Total n-6 PUFAs‡	0.15	0.02	0.09	0.13
18:2n-6 (LA)	-0.08	0.21	-0.04	0.57
20:4n-6 (AA)	0.13	0.04	0.09	0.13
n-3 PUFAs				
Total n-3 PUFAs§	-0.22	0.0003	-0.11	0.06
18:3n-3 (ALA)	-0.18	0.004	-0.13	0.04
20:5n-3 (EPA)	-0.24	<0.0001	-0.15	0.02
22:6n-3 (DHA)	-0.17	0.006	-0.06	0.31
6:3 Ratios				
Total n-6:n-3	0.23	0.0002	0.12	0.05
LA:ALA	0.18	0.004	0.14	0.03
AA:EPA+DHA	0.22	0.0004	0.11	0.07

NOTE: N=260. Fatty acids are reported as a percentage by weight of the total fatty acids (weight percent, wt. %). PUFA, polyunsaturated fatty acid; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid

*Unadjusted Spearman correlation coefficient

†Age- and BMI- adjusted Spearman correlation coefficient

‡18:2n-6+18:3n-6+20:2n-6+20:3n-6+20:4n-6+22:4n-6+22:5n-6

§18:3n-3+20:4n-3+20:5n-3+22:5n-3+22:6n-3

Table 4. Multivariable-adjusted geometric mean (95% CI) estradiol concentrations (pmol/L) by tertile of erythrocyte fatty acid according to NSAID use

Fatty Acids (wt. %)	N	All (N=253)	p for trend	N	NSAID Non-user (N=135)	p for trend	N	NSAID User (N=118)	p for trend
n-6 PUFAs									
Total n-6			0.17			0.02			0.44
≤37.32	83	16.7 (14.4, 19.3)		49	16.0 (13.4, 18.9)		34	18.5 (14.5, 23.7)	
37.33-39.49	86	16.0 (13.4, 18.9)		40	27.9 (23.2, 33.6)		46	18.8 (15.4, 23.1)	
≥39.50	84	16.0 (13.4, 18.9)		46	21.8 (18.3, 26.1)		38	16.3 (13.0, 20.5)	
18:2n-6 (LA)			0.73			0.99			0.66
≤14.69	83	19.3 (16.6, 22.2)		40	20.1 (16.4, 24.7)		43	17.8 (14.4, 22.1)	
14.70-16.84	86	20.8 (18.0, 23.9)		47	22.5 (18.7, 27.0)		39	19.3 (15.4, 24.1)	
≥16.85	84	18.5 (16.0, 21.3)		48	20.2 (16.8, 24.3)		36	16.6 (13.2, 20.9)	
20:4n-6 (AA)			0.19			0.09			0.95
≤15.24	83	18.8 (16.3, 21.7)		51	19.0 (16.0, 22.6)		32	18.8 (14.7, 24.0)	
15.25-16.57	86	18.3 (15.9, 21.0)		38	20.7 (16.8, 25.4)		48	16.6 (13.6, 20.2)	
≥16.58	84	21.5 (18.6, 24.9)		46	23.6 (19.6, 28.5)		38	18.9 (15.0, 23.8)	
n-3 PUFAs									
Total n-3			0.17			0.05			0.89
≤6.68	85	21.3 (18.5, 24.6)		46	24.3 (20.1, 29.3)		39	18.7 (14.9, 23.6)	
6.69-8.36	83	18.7 (16.2, 21.6)		45	20.4 (17.0, 24.6)		38	16.7 (13.3, 20.8)	
≥8.37	85	18.4 (16.0, 21.3)		44	18.4 (15.2, 22.3)		41	18.3 (14.6, 22.9)	

18:3n-3(ALA)			0.09			0.57		0.05
≤0.19	84	21.2 (18.4, 24.5)		42	21.5 (17.7, 26.2)		42	20.8 (16.8, 25.8)
0.20-0.25	86	19.5 (17.0, 22.4)		43	21.5 (17.7, 26.0)		43	17.5 (14.2, 21.5)
≥0.26	83	17.8 (15.4, 20.5)		50	20.0 (16.8, 23.9)		33	15.2 (12.0, 19.3)
20:5n-3 (EPA)			0.39			0.31		0.89
≤0.64	83	19.4 (16.8, 22.5)		48	20.9 (17.5, 25.1)		35	17.6 (13.8, 22.4)
0.66-0.90	85	21.4 (18.6, 24.6)		43	24.3 (20.0, 29.3)		42	18.9 (15.2, 23.3)
≥0.91	85	17.7 (15.4, 20.5)		44	18.2 (15.1, 21.9)		41	17.2 (13.8, 21.5)
22:6n-3 (DHA)			0.35			0.14		0.92
≤3.69	84	20.8 (18.0, 24.0)		48	23.1 (19.2, 27.8)		36	18.3 (14.4, 23.2)
3.70-4.91	84	18.9 (16.4, 22.0)		40	21.1 (17.4, 25.8)		44	17.0 (13.8, 20.9)
≥4.92	85	18.8 (16.3, 21.7)		47	18.8 (15.7, 22.7)		38	18.6 (14.7, 23.5)
6:3 Ratios								
Total n-6:n-3			0.21			0.06		0.98
≤4.48	84	17.7 (15.3, 20.3)		46	17.6 (14.6, 21.2)		38	17.7 (14.0, 22.4)
4.49-5.72	84	20.5 (17.8, 23.6)		43	22.9 (19.0, 27.7)		41	18.2 (14.6, 22.5)
≥5.73	85	20.3 (17.5, 23.4)		46	22.9 (19.0, 27.8)		39	17.8 (14.2, 22.3)
LA:ALA			0.03			0.51		0.01
≤64.00	85	17.6 (15.3, 20.3)		51	20.4 (17.0, 24.3)		34	14.2 (11.3, 17.9)
64.01-78.05	85	19.0 (16.5, 21.8)		46	20.5 (17.0, 24.7)		39	18.1 (14.6, 22.5)
≥78.06	83	22.1 (19.2, 25.5)		38	22.4 (18.2, 27.4)		45	21.1 (17.3, 25.8)
AA:EPA+DHA			0.18			0.01		0.63
≤2.68	84	18.6 (16.1, 21.5)		50	17.7 (14.8, 21.1)		34	19.9 (15.6, 25.5)
2.69-3.77	84	18.5 (16.1, 21.3)		41	21.5 (17.8, 26.0)		43	16.2 (13.2, 20.0)
≥3.78	85	21.4 (18.6, 24.7)		44	24.9 (20.6, 30.0)		41	18.1 (14.5, 22.7)

NOTE: Fatty acids are expressed as a percentage by weight of the total fatty acids (weight, percent, wt.%). Tertile cutpoints were determined from entire study population (n=260). NSAID, nonsteroidal anti-inflammatory drug; PUFA, polyunsaturated fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid. Values were adjusted for age (continuous), BMI (continuous), years menopausal (continuous), alcohol consumption (none, <12g/day, ≥12g/day; indicator variable), and current smoker (nonsmoker vs. smoker). 7 participants were excluded because years since menopause were undeterminable. p for interaction (fatty acid tertile x NSAID use) was significant for total n-6 ($p<0.02$) and suggestive for AA:EPA+DHA ($p<0.12$). Linear trend tests were performed by treating the fatty acid tertile groups as continuous variables

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Anti-inflammatory drug use and serum estradiol in postmenopausal women

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Epidemiologic data suggest a protective effect of anti-inflammatory drugs on the risk of breast cancer. These agents may exert their anti-tumorigenic effects by inhibiting cyclooxygenase (COX) enzyme activity, thereby reducing prostaglandin E2 (PGE2) production. PGE2 is an inflammatory mediator that induces estrogen biosynthesis, via upregulation of aromatase. The aim of the present study was to investigate the relationship between current anti-inflammatory drug use and serum estradiol levels in postmenopausal women. The study utilized data from a sample of 260 cancer-free postmenopausal controls enrolled in the Mammograms and Masses Study, a case-control study in Pittsburgh, PA. Medication use was determined by self-report. Participants were excluded from this analysis if they were using exogenous hormones, antiestrogens or corticosteroids at blood draw. Serum estradiol (E2) and sex hormone binding globulin (SHBG) concentrations were measured by indirect radioimmunoassay. Analysis of covariance was used to estimate differences in mean estradiol levels between groups (user vs. nonuser) adjusting for age, body mass index, and SHBG. Hormone levels were logarithmically transformed and geometric means are presented. 137 women (52.7%) reported current use of COX-2 inhibitors, aspirin and/or non-aspirin nonsteroidal anti-inflammatory drugs (NSAIDs). Current users of anti-inflammatory agents had lower E2 levels than nonusers with adjusted mean levels of 17.46 and 21.88 pmol/L, respectively ($P = 0.008$). Further adjustment did not appreciably alter results. Our findings suggest that anti-inflammatory agents may be useful in decreasing circulating estradiol levels in postmenopausal women.

Nonsteroidal Anti-inflammatory Drug Use and Serum Total Estradiol in Postmenopausal Women

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Abstract

Laboratory and epidemiologic evidence suggest that nonsteroidal anti-inflammatory drug (NSAID) use may be inversely related to the risk of breast cancer; however, the mechanism by which NSAIDs may protect against the development of this disease is uncertain. The objective of this observational study was to assess the relationship between current NSAID use and endogenous estradiol levels, an established breast cancer risk factor. To evaluate this aim, we conducted a cross-sectional investigation among 260 postmenopausal women who were not recently exposed to exogenous hormones. Information on current NSAID use (aspirin, cyclooxygenase-2 inhibitors, and other NSAIDs combined) was collected using a questionnaire at the time of blood draw. Estradiol was quantified in serum by radioimmunoassay. General linear models were used to evaluate the association between NSAID use and serum total estradiol. The age-adjusted and body mass index–adjusted geometric mean serum estradiol concentration among NSAID users (n = 124) was significantly lower than nonusers of NSAIDs (n = 136; 17.8 versus 21.3 pmol/L; P = 0.03). Further adjustment for additional potential confounding factors did not substantially alter estimates (17.7 versus 21.2 pmol/L; P = 0.03). To our knowledge, this report is the first to examine the relationship between NSAID use and serum estradiol in postmenopausal women. These cross-sectional findings suggest that NSAID use may be associated with lower circulating estradiol levels, potentially representing one mechanism through which NSAIDs exert protective effects on breast cancer. (Cancer Epidemiol Biomarkers Prev 2008;17(3):680–7)

Introduction

Although breast cancer is a major public health problem, little is known about preventing this disease. Experimental studies have reported a protective effect of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, against mammary carcinogenesis (1-3), and accumulating evidence from both case-control and cohort studies suggests that use of NSAIDs may be associated with a modest decreased risk of breast cancer in women (4-10). However, findings are mixed (11-17). Clarifying the association between NSAID use and the development of breast cancer is potentially of great importance clinically. NSAIDs are widely used, readily available, and inexpensive agents. If they were shown to be chemopreventive, they could have a substantial effect on public health.

Although the mechanisms by which NSAIDs may protect against breast cancer are not fully understood, data suggest that the protective effect may be attributed in part to the ability of NSAIDs to decrease the formation of prostaglandin E_2 (PGE_2) by blocking cyclooxygenase (COX)-1 and/or COX-2 activity. One possible mechanism by which the COX/PGE_2 cascade promotes breast cancer is via increasing estrogen production, as exposure to endogenous estrogens has been shown to play a causal role in the development of some breast cancers (18).

PGE_2 up-regulates aromatase activity (19), the enzyme that converts androgens to estrogens, leading to increased estrogen synthesis. In postmenopausal women, aromatic conversion of androgens is the primary source of circulating estrogens, and suppression of this enzyme has been shown to have a profound effect on both circulating estrogen levels (20) and breast cancer recurrence (21). Recently, dose-dependent decreases in aromatase activity were observed in breast cancer cells following treatment with NSAIDs, a COX-1 selective inhibitor, and COX-2 selective inhibitors (22). Therefore, NSAIDs may offer protection against breast cancer by reducing a woman's exposure to estrogen via the inhibition of aromatase activity. Indeed, laboratory results have shown that estradiol production is decreased in breast cells that are exposed to the selective COX-2 inhibitor celecoxib (23).

Although the above-mentioned pathway through which NSAIDs may decrease the development of breast cancer has been highlighted previously (24, 25), the association between NSAID use and circulating estradiol

Received 10/27/07; revised 12/13/07; accepted 12/21/07.

Grant support: NIH grants R25-CA57703, K07-CA80668, R21-CA95113, and P20 CA103730-02; Department of Defense grants DAMD17-02-1-0553 and W81XWH-06-1-0532; and Pennsylvania Department of Health grant P2777693. Additional support was provided by funds received from the NIH/National Center for Research Resources/General Clinical Research Center grant MO1-RR000056.

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doi:10.1158/1055-9965.EPI-07-2739

in women is currently unknown. Therefore, in this cross-sectional investigation, we asked whether differences in serum estradiol levels could be observed between self-reported NSAID users and nonusers in a population of postmenopausal women not taking menopausal hormone therapy.

Materials and Methods

Study Population. We used data from controls drawn from the Mammograms and Masses Study (MAMS), a case-control study of estrogen metabolites, mammographic breast density, and breast cancer risk. Details of the study methodologies have been presented elsewhere (26). In brief, 869 cancer-free women and 264 recently diagnosed breast cancer cases were recruited into the MAMS through the Magee-Womens Hospital Mammographic Screening and Diagnostic Imaging Program in the greater Pittsburgh area between September 2001 and May 2005. Women who were ages ≥18 years, who reported no previous personal history of cancer, with the exception of nonmelanoma skin cancer, and who could provide written informed consent were eligible for study enrollment. Participants in the MAMS include (*a*) breast cancer cases who were recruited from the Magee-Womens Surgical Clinic for an initial evaluation after newly diagnosed primary breast cancer (n = 264), (*b*) controls who were undergoing outpatient needle breast biopsy through the Breast Biopsy Service at Magee-Womens Hospital but who were not subsequently diagnosed with breast cancer (n = 313), (*c*) ''healthy'' controls who received screening mammography through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers (n = 538), and (*d*) an additional 18 participants whose blood was dedicated solely to an ancillary study of intraindividual cytokine and hormone level reproducibility. To increase recruitment of the ''healthy'' control group, study flyers were attached to screened negative mammogram reports mailed to patients between November 2003 and April 2005. The MAMS is approved by the University of Pittsburgh's Institutional Review Board and all participants provided written informed consent at the time of study entry.

Subsample Selection. Participants were selected for the present study if they met the following eligibility criteria: (*a*) healthy controls recruited only via study flyers through Magee-Womens Hospital or through Pittsburgh Magee Womancare Centers (n = 453), because these participants completed a self-administered questionnaire on the day of blood draw; (*b*) postmenopausal, defined as having no menstrual bleeding during the year before enrollment, having undergone a bilateral oophorectomy, or having a hysterectomy without bilateral oophorectomy and ages ≥50 years. We measured follicle-stimulating hormone for women ages <55 years at blood draw who had a hysterectomy without bilateral oophorectomy (n = 5); all five participants had follicle-stimulating hormone levels above 40 mIU/mL (range, 49.1-185.2), consistent with follicle-stimulating hormone elevation in the postmenopausal range (27); (*c*) did not use hormone therapy within 3 months of enrollment; and (*d*) did not report using vaginal estrogen creams, oral contraceptives, selective estrogen receptor modulators, or corticosteroids on the day of blood draw. Ninety-eight premenopausal women, 55 postmenopausal women using hormone therapy, 24 women using selective estrogen receptor modulators, 5 participants on corticosteroids, and 1 participant later found to have a personal history of breast cancer were excluded from the study. Two hundred and seventy participants met the above-mentioned criteria.

Covariate Information. A standardized, self-administered questionnaire was used to gather exposure information. Participants in the subsample completed the questionnaire at study enrollment on the day of blood draw. Information collected included demographic data, current use of medication and supplements, reproductive history, family medical history, past exogenous hormone use, and lifestyle factors, such as smoking status and alcohol intake. Alcohol use (g/d) in the past year was calculated as reported previously (28). Age at onset of menopause was defined according to the methods formerly described by the Women's Health Initiative (29), where age at menopause corresponded to the age of a woman's last natural menstrual bleeding, bilateral oophorectomy, or age a woman began using hormone therapy. For a hysterectomized woman without a bilateral oophorectomy, age at menopause was the earliest age at which she began using hormone therapy or first had menopausal symptoms. If neither occurred and her age at hysterectomy was ≥50 years, then age at menopause was her age at hysterectomy. Age at menopause was undeterminable in seven participants. Years since menopause were calculated by subtracting the age at menopause from the age at enrollment.

Assessment of NSAID Use. The primary exposure variable ''current NSAID use'' was collected on the day of blood draw. On the self-administered questionnaire, participants were asked to report all prescribed and over-the-counter medications that were currently being used. The question asked, ''Are you CURRENTLY taking any medications (prescription or over-the-counter, including aspirin and ibuprofen)?'' If a participant responded affirmatively, she was prompted to ''please list them in this table.'' Dosage data were collected but not analyzed as many participants knew only the number of tablets taken rather than the actual dose. The questionnaire was reviewed for completeness by a trained research nurse (study coordinator), who queried participants if further clarification was needed. Each medication reported in the table was subsequently assigned a code using a therapeutic classification system as indexed in the Nurse Practitioners' Prescribing Reference, which is updated quarterly (30). Participants who listed aspirin, COX-2 inhibitor, or other non-aspirin NSAID use on the questionnaire were considered ''current NSAID users.'' Participants who did not list using a NSAID were considered ''current NSAID nonusers.'' Because acetaminophen is generally reported to be a poor inhibitor of the COX-1/COX-2 enzymes (31) and its mechanism of action has yet to be resolved, we classified acetaminophen users as nonusers of NSAIDs (n = 12) unless they also reported taking a NSAID (n = 6).

Two additional NSAID exposure variables were considered in relation to estradiol levels, a secondary exposure variable and a NSAID variable constructed from the primary and secondary variables. The secondary

NSAID exposure variable was from the participant's yes-or-no response to the study phlebotomist's question at blood draw, ''Have you taken any aspirin or anti-inflammatory agents in the last 48 h?'' No effort was undertaken to determine the specific agent the participant had used. Therefore, this variable is more subjective in that responses were based solely on each individual's perception of what constitutes an anti-inflammatory agent and aspirin. The secondary exposure variable was used in conjunction with the primary NSAID exposure variable to construct a third variable labeled ''consistent NSAID use.'' ''Consistent NSAID users'' listed on the questionnaire that they were currently taking a medication that was an aspirin, COX-2 inhibitor, or non-aspirin NSAID and also verbally reported that they took an aspirin or other anti-inflammatory agent in the past 48 h. ''Consistent NSAID nonusers'' did not list using any NSAID nor did they state having taken an aspirin or anti-inflammatory agent in the past 48 h. This latter variable was created as an attempt to reduce potential NSAID use/nonuse misclassification. None of the participants in this analysis were missing any of the NSAID exposure variable data. Exposure data were collected and coded without knowledge of estradiol levels.

Clinical Measures. The study coordinator obtained physical measurements (height and weight) and recorded information on a standardized form. After the participant removed her shoes and heavy clothing, weight was measured at a standing position to the nearest 0.1 kg using a standard balance beam; standing height was measured at full inspiration to the nearest 0.1 cm. All anthropometric measurements were taken twice and were repeated if the first two measurements differed by more than 0.5 cm or 0.5 kg. The mean of the measurements was used in the analysis. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2).

Forty milliliters of peripheral nonfasting blood were collected from the participants at study enrollment. All samples were processed on site at the Magee-Womens Hospital Satellite General Clinical Research Center according to standard protocols. After processing, the samples were aliquoted into 1 mL cryovials in which RBC, serum, plasma, and buffy coat were separated. Samples were stored at or below -70°C before laboratory analyses.

Laboratory Analyses. Serum samples were used for the quantification of total estradiol (sex hormone binding globulin and albumin-bound plus unbound estradiol) and were assayed at the Royal Marsden Hospital in England. Estradiol concentrations were measured by RIA after ether extraction, using a highly specific rabbit antiserum raised against an estradiol-6-carboxymethyloxime-bovine serum albumin conjugate (EIR) and Third-Generation Estradiol [I125] reagent DSL 39120 (Diagnostic Systems Laboratories; ref. 32). The assay detection limit was 3 pmol/L by calculation from the 95% confidence limits of the zero standard. A random subset of 27 replicate quality-control samples was included to assess reproducibility; the calculated coefficient of variation between duplicates for estradiol was 14.5%. Laboratory personnel were masked to both subject identification and quality-control status.

Statistical Analyses. Wilcoxon's rank-sum test was used to compare selected continuous characteristics between current users and nonusers of NSAIDs and the χ^2 test or the Fisher's exact test was used to assess differences in categorical variables. The Kruskal-Wallis test was used to test for significant differences in continuous characteristics across estradiol tertile categories. A log transformation was applied to serum estradiol concentrations to obtain homoscedacity and an approximately normal distribution for linear model residuals. One participant was excluded from analyses because total estradiol levels were deemed unreliable by the laboratory. An additional nine participants with outlying estradiol values, defined as >2 SD from the mean of estradiol concentration (>150 pmol/L; range, 150-847 pmol/L), were removed from analyses because such high levels likely indicated that the women were not postmenopausal or did not correctly report current hormone use. Thus, the final analytic subsample included 260 women.

Cohen's κ statistic was calculated as a measure of agreement between the primary and secondary NSAID exposure variables. Differences in mean log estradiol levels between users and nonusers of NSAIDs were tested by the Student's t test. The general linear model approach was performed to calculate multivariable-adjusted estradiol levels and to assess differences in levels between NSAID users and nonusers. Adjusted means and confidence intervals for each NSAID category were quantified using the least-squares mean option of PROC GLM. Two adjusted models are presented. The first model was adjusted for age and BMI, which were deemed necessary covariates given their previously reported associations with both NSAID use (33) and estradiol levels (34, 35). The second model was further adjusted for variables found to be associated with NSAID use or estradiol levels within the study population (univariate association $P < 0.15$). The final multivariable model was adjusted for age (continuous), BMI (continuous), years since menopause (continuous), race (White versus non-White), and regular alcohol intake in the past year (none, <12 g/d, ≥12 g/d, entered as an indicator variable). Additional adjustment for family history of breast cancer, past hormone therapy use, smoking status, sex hormone binding globulin, and various reproductive factors yielded similar results and are not presented. The geometric mean estradiol concentrations were calculated by taking the anti-log of the least-squares means after adjustment. For each model, a plot of the studentized residuals versus the predicted values was examined to check whether the equality of variance assumption was met. A normal probability plot of the residuals was examined to assess normality. Assumptions of normality and homogeneity of variance were met for all models presented. Tests of statistical significance were two tailed, and given the exploratory nature of this work, we reported our results at the $P < 0.05$ significance level rather than correct for multiple comparisons. All analyses were done using SAS software version 9.1 (SAS Institute).

Results

Characteristics of the study population are shown in Table 1. The majority of participants (66.9%) were

Table 1. Distribution of selected characteristics by NSAID use among 260 postmenopausal women in the MAMS

Characteristic	Current NSAID use		*P*
	User (n = 124)	Nonuser (n = 136)	
Age at blood draw (y), mean (SD)	62.6 (8.1)	62.9 (8.7)	0.91
BMI (kg/m^2), mean (SD)	28.6 (6.0)	28.3 (6.1)	0.63
Years menopausal, mean (SD)*	14.1 (9.8)	14.2 (10.3)	0.94
Race, %			
White	96.8	89.7	0.03
Non-White	3.2	10.3	
Regular alcohol intake in past year, %			
None	66.1	77.9	0.10
<12 g/d	21.8	14.0	
≥12 g/d	12.1	8.1	

*Missing n = 7 for years menopausal.

overweight or obese (BMI ≥ 25 kg/m^2) and White (93.1%). Overall, 124 (47.7%) participants reported current NSAID use at the time of blood draw (Table 2). In this study, 25.0%, 12.3%, and 2.3% participants reported using only aspirin, non-aspirin NSAIDs, and COX-2 selective inhibitors, respectively, whereas 8.1% reported using at least two different types of NSAIDs (data not shown). One hundred forty (53.8%) women reported that they took aspirin or another anti-inflammatory agent within 48 h of blood draw. One hundred (38.5%) participants listed current use of a NSAID on the baseline questionnaire and verbally reported aspirin or anti-inflammatory use within 48 h of blood draw, and 96 (36.9%) reported no use of NSAIDs in both settings. The agreement between the primary and secondary exposure variables was moderate with a κ value of 0.51.

With the exception of race, current NSAID users and nonusers were statistically similar with regard to all other demographic characteristics (Table 1). Current users of NSAIDs were more likely to be White than nonusers (96.8% versus 89.7%; P = 0.03). Demographic differences between users and nonusers for all NSAID exposure variables (primary, secondary, and constructed) were similar, with the exception of BMI. Participants who reported aspirin or anti-inflammatory drug use within the past 48 h and those who were consistent users not greater BMIs than participants who reported no use (data not shown).

The geometric mean serum estradiol concentration for the study population was 19.5 pmol/L, with levels ranging from 3.3 to 140.0 pmol/L. As illustrated in Table 3, higher serum estradiol levels were associated with increasing BMI (P < 0.0001) and negatively associated with alcohol intake (P = 0.003). Although not statistically significant, it was observed that women with higher circulating estradiol levels were on average fewer years from menopause (P = 0.11). With the exception of alcohol intake, all associations persisted after controlling for BMI (data not shown). The association between alcohol intake and estradiol diminished after controlling for BMI.

After adjustment for age and BMI, current NSAID use was significantly inversely associated with serum estradiol concentrations (17.8 versus 21.3 pmol/L; P = 0.03; Table 4), with ~16.4% lower levels in users than nonusers of NSAIDs. The age- and BMI-adjusted association between use of the secondary NSAID exposure variable (aspirin or anti-inflammatory agent in the past 48 h) and estradiol was suggestive of an inverse effect, but this finding was not statistically significant (18.5 versus 20.9 pmol/L; P = 0.14). A slightly stronger association between NSAID use and estradiol levels was observed when comparing consistent users with consistent nonusers (17.5 versus 21.5 pmol/L; P = 0.03). Further adjustment for race, alcohol intake, and years menopausal only slightly increased the strength of association observed in the age- and BMI-adjusted analyses.

Figure 1 presents the adjusted geometric mean serum estradiol concentration by subcategory of NSAID use as defined by the cross-tabulation of the primary and secondary NSAID exposure variables. Three categories were defined, the two concordant groups (that is, ''No NSAIDs on medication list/No NSAIDs verbally'' and ''Yes NSAIDs on medication list/Yes NSAIDs verbally'') remained as separate exposure categories, whereas the two discordant groups (that is, ''No NSAIDs on medication list/Yes NSAIDs verbally'' and ''Yes NSAIDs on medication list/No NSAIDs verbally'') were collapsed into a single category. The three groups had significantly

Table 2. Self-reported NSAID use in the MAMS

NSAID use	n (%)
Primary exposure variable	
Current use*	
Nonuser	136 (52.3)
User	124 (47.7)
Secondary exposure variable	
Past 48 h use†	
Nonuser	120 (46.2)
User	140 (53.8)
Constructed exposure variable	
Consistent use‡	
Nonuser	96 (36.9)
User	100 (38.5)

*Current use: Based on participants' self-reported current medication list.
† Past 48 h use: Based on participants' verbal response to the question: ''Have you taken an aspirin or other anti-inflammatory drug in the past 48 h?''
‡ Consistent use: The agreement between current use and past 48 h use. Nonuser = Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that she did not consume an aspirin or anti-inflammatory agent within 48 h of blood draw. User = Participant's current medication list indicated use of a NSAID and the participant verbally responded that she consumed an aspirin or anti-inflammatory agent within 48 h of blood draw.

Table 3. Distribution of selected characteristics by tertile of serum estradiol concentration among 260 postmenopausal women in the MAMS

Characteristic	Estradiol concentrations			*P*
	Tertile 1 (n = 91)	Tertile 2 (n = 81)	Tertile 3 (n = 88)	
Age at blood draw (y), mean (SD)	63.8 (8.5)	62.6 (8.5)	61.9 (8.2)	0.38
BMI (kg/m^2), mean (SD)	25.4 (4.4)	27.3 (4.9)	32.6 (6.2)	<0.0001
Years menopausal, mean (SD)*	15.6 (9.7)	13.5 (10.2)	13.1 (10.1)	0.11
Race, %				
White	95.6	92.6	90.9	0.46
Non-White	4.4	7.4	9.1	
Regular alcohol intake in past year, %				
None	60.4	72.8	84.1	0.003
<12 g/d	28.6	13.6	10.2	
≥12 g/d	11.0	13.6	5.7	

*Missing n = 7 for years menopausal.

different adjusted mean estradiol levels (P_{trend} = 0.02). Mean estradiol was lowest for participants who reported NSAID use for both measures and highest for participants who did not report use for either measure.

To assess the possible effects of acetaminophen use on the findings, all analyses were repeated, excluding acetaminophen users from the NSAID nonuser groups. Results did not differ substantially (data not shown). We also assessed unadjusted and adjusted relationships between all NSAID exposure variables and log-transformed sex hormone binding globulin, but no statistically significant relationships were observed (data not shown).

Discussion

In this cross-sectional investigation, we observed lower circulating estradiol levels among postmenopausal women reporting NSAID use. Specifically, we observed ~16% lower estradiol levels among current users than nonusers. Decreased estradiol levels were consistent regardless of how NSAID use was assessed (that is, self-reported current NSAID use on questionnaire, verbal reporting of use in past 48 h, and the agreement between these two variables). Further, the strength of association was slightly stronger when comparing participants who reported NSAID use at both the time of blood draw and within 48 h of blood draw to those who reported no use of NSAIDs for both measures. Associations were independent of age, BMI, and other potential confounding variables. As elevated serum estradiol levels have been linked to breast cancer risk, these results provide support to the growing body of evidence linking NSAID use to decreased breast cancer incidence.

Although findings in the literature are not completely consistent, results of several epidemiologic studies

Table 4. Unadjusted and adjusted geometric mean (95% CI) estradiol concentrations according to categories of NSAID use

NSAID use	Serum estradiol concentrations (pmol/L)					
	Model 1*	*P*	Model 2†	*P*	Model 3‡	*P*
Primary exposure variable						
Current use		0.11		0.03		0.03
Nonuser (n = 136)	21.0 (18.4-24.0)		21.3 (19.0-23.7)		21.2 (18.9-23.7)	
User (n = 124)	18.0 (15.7-20.7)		17.8 (15.9-20.0)		17.7 (15.7-19.9)	
Secondary exposure variable						
Past 48 h use		0.94		0.14		0.07
Nonuser (n = 120)	19.5 (16.9-22.4)		20.9 (18.5-23.5)		21.1 (18.7-23.8)	
User (n = 140)	19.6 (17.2-22.3)		18.5 (16.5-20.6)		18.1 (16.2-20.3)	
Constructed exposure variable						
Consistent use		0.39		0.03		0.02
Nonuser (n = 96)	20.3 (17.3-23.8)		21.5 (18.9-24.4)		21.4 (18.8-24.4)	
User (n = 100)	18.4 (15.8-21.5)		17.5 (15.4-19.8)		17.2 (15.1-19.6)	

Current use: Based on participant's self-reported current medication list. Past 48 h use: Based on participant's verbal response to the question ''Have you taken an aspirin or other anti-inflammatory drug in the past 48 h?'' Consistent use: The agreement between current NSAID use and past 48 h use. Consistent Nonuser = Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that she did not consume an aspirin or anti-inflammatory agent within 48 h of blood draw. Consistent User = Participant's current medication list indicated use of a NSAID and the participant verbally responded that she consumed an aspirin or anti-inflammatory agent within 48 h of blood draw.

*Unadjusted model.

† Adjusted for age at blood draw (continuous) and BMI (continuous).

‡ Missing n = 7; adjusted for age at blood draw (continuous), BMI (continuous), race (White, non-White), years menopausal (continuous), and current alcohol intake (none, <12 g, ≥12 g, indicator variable).

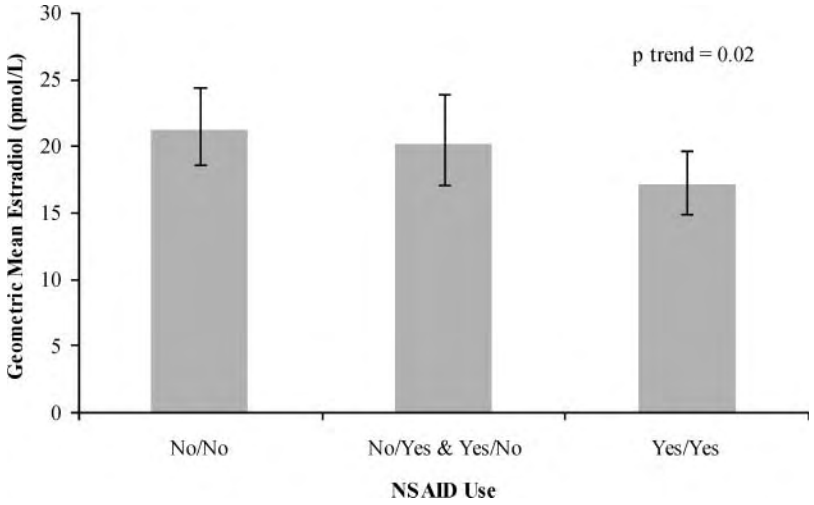

Figure 1. Adjusted geometric mean (95% CI) of estradiol according to self-reported NSAID use. Serum total estradiol was adjusted for age at blood draw, BMI, race, years menopausal, and current alcohol intake in a general linear model (n = 7 missing data). No/No = Participant's current medication list did not indicate use of a NSAID and the participant verbally responded that she did not consume an aspirin or anti-inflammatory agent within 48 h of blood draw. No/Yes = Participant's current medication list did not indicate use of a NSAID but participant verbally responded that she consumed an aspirin or anti-inflammatory agent within 48 h of blood draw. Yes/No = Participant's current medication list indicated use of a NSAID but the participant verbally responded that she did not consume an aspirin or anti-inflammatory agent within 48 h of blood draw. Yes/Yes = Participant's current medication list indicated use of a NSAID and the participant verbally responded that she consumed an aspirin or anti-inflammatory agent within 48 h of blood draw.

suggest that use of NSAIDs may reduce the risk of breast cancer (reviewed in ref. 36). The inconsistent findings across studies may be explained in part by differences in the definition of NSAID use, dosage and frequency data, and NSAID assessment periods. Notably, some studies suggest the decreased risk is stronger among estrogen receptor–positive breast cancers (37, 38) and, if true, would strengthen the hypothesis of an estrogen modulatory effect by NSAIDs. However, this relationship is not consistently observed (10, 39).

The reduced risk of breast cancer observed among NSAID users in epidemiologic studies may in part be mediated through the favorable effects of NSAIDs on PGE_2 production. Decreased PGE_2 synthesis may result in suppressed estradiol production in postmenopausal women and subsequently reduced breast cancer risk. In accordance with this biological paradigm, we observed that postmenopausal participants reporting NSAID use had lower estradiol levels. Therefore, this study adds credence to the epidemiologic data illustrating a protective effect between NSAID use and breast cancer incidence. As NSAID use is modifiable, a chemoprotective action attributed to its use could have a considerable public health effect. However, the risk-to-benefit ratio would need to be considered because NSAIDs have potentially serious side effects (40, 41).

The present study has limitations that deserve attention and that should be considered when evaluating the study findings. First, as this is a cross-sectional investigation, we cannot ascertain the temporal relationship between NSAID use and serum estradiol and causal conclusions cannot be made. Multiple measurements of NSAID use and serum estradiol may have resulted in more precise estimates. Additional limitations of this study include our inability to assess duration of NSAID use or dosage information, as duration of NSAID use was not collected and dosage data were deemed unreliable. Women exposed to a longer duration of NSAID use or larger doses may have more pronounced effects on circulating estradiol levels than occasional NSAID users (that is, as-needed) or those consuming smaller doses (that is, low-dose aspirin).

The sample size was not large enough to assess the effects of the different NSAIDs (e.g., aspirin and selective COX-2 inhibitors) on estradiol levels. Further, we cannot rule out exposure misclassification. The result of nondifferential misclassification of our exposure variable (NSAID use versus NSAID nonuse) would most likely bias the findings toward the null hypothesis and possibly underestimate the true association between NSAID use and serum estradiol. We attempted to reduce misclassification by repeating analyses limiting the sample to women who consistently reported NSAID use or nonuse. Further, although we attempted to control for potential confounders in the statistical analyses, we cannot rule out the possibility that women who are users of NSAIDs had a factor in common that we did not measure that is related to lower serum estradiol levels. Contrary to our findings, previous studies have observed positive associations between alcohol intake and postmenopausal endogenous estradiol levels; however, our results are consistent with others observing no association between alcohol and estradiol levels after adjustment for BMI (42). Finally, the lack of ethnic diversity and exclusion of premenopausal women in our sample limits the generalizability of the results.

Strengths of our study include the use of standardized instruments, reproducible measures of total estradiol, and the assessment of NSAID use on the same day as blood draw. The last strength is important, because the effect of NSAIDs on the inhibition of COX enzymes and PGE_2 synthesis occurs rapidly (43). Finally, the observed distribution of postmenopausal total estradiol levels and the self-reported prevalence of NSAID use in this population were similar to previous reports (35, 44).

In summary, we believe this to be one of the first reports on the association between NSAID use and postmenopausal estradiol levels. We found NSAID users to have significantly lower serum estradiol than nonusers which may account for the protective effect NSAID use has been observed to exhibit on breast cancer development. However, continued research efforts are needed to verify our findings.

Acknowledgments

We thank Glenn Allen, Katherine Reeves, Betty Kotowski, and Elizabeth Wentzel for contributions to MAMS and Dr. Mitch Dowsett and Dr. Elizabeth Folkerd and Ms. Debbie Doody for conducting the serum estradiol assays.

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